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Impact of DNA Damage on the Frequency of Sperm Chromosomal Aneuploidy in Normal and Subfertile Men

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ABSTRACT

Background: Various frequencies of sperm aneuploidy are reported in sperms of subfertile patients compared to normal individuals. Moreover, sperm DNA damage is shown to be associated with male infertility. In this study, the rate of DNA damage and frequencies of aneuploidy in sperms of subfertile patients was investigated. **Methods:** Semen samples were obtained from healthy normal and subfertile (oligozoospermia, asthenozoospermia, and oligoasthenozoospermia) men. The frequency of aneuploidy was assessed using primed *in situ* labeling (PRINS) analysis with specific primers for chromosomes 18, 21, X, and Y. Sperm DNA damage was assessed using alkaline comet assay. **Results:** The mean frequencies of disomy for the patients were significantly higher than normal for all chromosomes (P<0.01). The extent of DNA damage in sperms of subfertiles was significantly higher than in normal individuals (P<0.001). The obtained results indicated that higher rate of DNA damages led to higher frequency of chromosomal disomy except for asthenozoospermia samples which exhibited higher rate of DNA damage and lower frequency of chromosomal disomy. **Conclusions**: These results demonstrate that men with oligozoospermia and oligoasthenozoospermia have an elevated risk for chromosome abnormalities in their sperm, particularly sex chromosomes. DNA damage might be involved in the process of malsegregation of chromosomes. *Iran. Biomed. J. 15 (4): 122-129, 2011*

Keywords: Asthenozoospermia, DNA damage, Primed *in situ* labeling (PRINS), Comet assay

INTRODUCTION

Sperm nuclear defects might be the reason for a worldwide increasing trend of male infertility in terms of average low sperm counts and sperm quality in developed countries [1]. Sperm cells carry a demonstrable background level of aneuploidy and chromosome breakage [2, 3]; however, a number of risk factors might lead to increase this baseline.

The causes of sperm DNA damage, much like those of male infertility, have many factors and may be attributed to interior extra testicular factors (i.e. drugs, chemotherapy, radiation therapy, cigarette smoking, environmental toxins, genital tract inflammation, testicular hyperthermia, varicocele, hormonal factors and so on). Sperm DNA damage is clearly associated with male infertility (and abnormal spermatogenesis), but a small percentage of spermatozoa from fertile men also possesses detectable levels of DNA damage [4].

In addition, sperm chromosome abnormalities may have a negative impact on the success rate of assisted reproduction technology (ART) program. Indeed, a higher incidence of sex chromosome aneuploidy has been reported in subfertile individuals who needed intracytoplasmic sperm injection (ICSI) [2] compared with men requiring conventional IVF [5].

Although ICSI has become a method of choice for the treatment of male infertility, but there is some concern that infertile men may have a higher frequency of chromosomal abnormalities in their sperm. Therefore, the risk of chromosomally abnormal offspring may increase in patients using ICSI for treatment. Some studies have shown that infertile men with normal somatic karyotypes have an increased risk of aneuploid sperm [6]. Also, an inverse correlation between sperm aneuploidy rate and conventional semen parameters (sperm density, motility and morphology) has been reported [7]. Therefore, patients who are more likely to undergo ICSI cycles for their infertility, also have the greatest proportion of aneuploid sperm.

Several techniques have been developed to detect

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individual human chromosomes in sperm nuclei. The first preparation of human sperm chromosomes was done using the capacity of human spermatozoa to penetrate zona-free hamster oocytes [8]. This method was standardized by Martin e*t al.* [9], but the technique was so complex and time-consuming that its use was limited to a few laboratories, and was never applied in a clinical setting. The use of chromosome-specific DNA probes opened the way to indirectly study the chromosome constitution of large numbers of spermatozoa by multi-color fluorescence *in situ* hybridization (FISH) either on sperm nuclei or on human sperm-derived pronuclei [3, 4, 10]. However, the special characteristics of the sperm nucleus (compact packaging with protamine) give rise to a series of limitations that can only be circumvented through the use of very strict technical criteria. The primed *in situ* labeling (PRINS) technique has provided an interesting alternative to sperm FISH method and represents a significant improvement in the detection of specific DNA sequences *in situ*; it is based on the annealing of specific oligonucleotide primers to chromosomal DNA and subsequent primer extension by a suitable polymerase in the presence of labeled nucleotides [11-13]. Compared to FISH, this method does not use large pieces of probes; instead it uses short primers with high accessibility to the specified targets in DNA. PRINS is also faster to perform and cheaper compared to FISH [11-13]. To date, there are only a few reports of such a study on sperms of subfertile patients using PRINS technique. In this work, we studied the frequency of disomy in 44 Iranian normal and subfertile individuals for two autosomes (chromosomes 18 and 21) and sex chromosomes by the PRINS technique. The frequency of aneuploidy observed for each chromosome was then correlated to DNA damage assessed using the alkaline comet assay for each study group. The comet assay, originally known as the single-cell gel electrophoresis assay, has been shown to be an effective indicator test for germ cell genotoxicity [14] and prognostic test for male infertility and assisted reproductive technology outcomes [15]. The alkaline comet assay, originally known as the single cell gel electrophoresis assay, assesses actual DNA strand breaks and alkaline-labile sites when used under alkaline condition. The comet assay is already recognized as being among the most sensitive methods available for measuring DNA strand breaks; it has further advantages of speed, reproducibility, simplicity, and the fact that observations are made at the level of single cells. The alkaline comet assay can detect damage equivalent to as few as 50 single-strand breaks per cell [14, 15]. Baseline DNA damage in spermatozoa in most of the published studies with the comet assay is significantly higher than in somatic cells. The nature of a high background level of DNA damages in spermatozoa might be due to natural physiological events leading to DNA damage induction, such as production of reactive oxygen species (ROS) [16] and normal differentiation program [17].

MATERIALS AND METHODS

Sample preparation. Semen samples were obtained randomly from 14 normal and 30 subfertile men referred to Fertility and Infertility Center of Shariati Hospital (Tehran, Iran), candidate for ART. In all cases, after three days of sexual abstinence, semen samples were collected by masturbation into sterile containers and were delivered to the laboratory immediately after ejaculation. The semen was allowed to stand at 37°C for 30 min to complete liquefaction. After semen analysis, samples were classified according to the World Health Organization criteria [18] into a normal and three subfertile groups (oligozoospermia, asthenozoospermia and oligo-asthenozoospermia). Samples considered as normal were obtained from men without any male fertility problems. These individuals referred for ART because of fertility problems of their spouse. Some demographic characteristics of the study groups are shown in Table 1. The study was approved by the Ethical Committee of the School of Medical Sciences of the Tarbiat Modares University (Tehran, Iran). Patients gave their informed written consent and all donors completed a written questionnaire to obtain information related to their life style, such as dietary habits, medical history and exposure to chemical and physical agents. Therefore, all samples had been screened to exclude radiation exposure, smoker, varicocele, genital tract infections, hepatitis, and HIV. Samples were processed by swim-up techniques from pellet as described by Aitken and Clarkson [19].

Table 1. Demographic characteristics of the study groups.

Study group	n	Mean age ± SD (Years)	Mean sperm concentration ± SD ($\times 10^6$/ml)	Mean period of Infertility ± SD (Years)
Normal	14	33.3 ± 5.6	68.6 ± 20.4	6.6 ± 5.3
Oligozoospermia	10	34.1 ± 7.9	12.4 ± 2.7	8.5 ± 6.6
Asthenozoospermia	10	34.5 ± 5.5	42.1 ± 18.0	5.9 ± 2.5
Oligoasthenozoospermia	10	34.4 ± 6.9	9.2 ± 4.0	6.5 ± 4.4

Table 2. Primer sequences used in PRINS reactions for detection of desired chromosomes (adopted from Pellestor *et al.* [19]).

Chromosome	Primer	Annealing temp. (˚C)
18	5'-ATGTGTGTCCTCAACTAAAG-3'	65
21	5'-TGATGTGTGTACCCAGCC-3'	61
X	5'-GTTCAGCTCTGTGAGTGAAA-3'	68
Y	5'-TCCATTCGATTCCATTTTTTTCGAGAA-3'	56

Sperm comet assay. Briefly, alkaline comet assay was performed based on existing methods described by Singh [20] with minor modifications [21]. Observations were made at a magnification of ×200 using a Nikon E800 epifluorescence microscope (Japan) equipped with 546-516 wave length band and a 590 nm barrier filter. The comets were analyzed by visual classification [22] and for each sample, 1,000 cells were scored. Damage was assigned to five classes (0-4) based on the visual aspect of the comets (Fig. 1), considering the extent of DNA migration according to the established criteria [21]. Damage scores were calculated based on the following equation adopted from Jaloszynski *et al.* [23] that ranged from 0 to 400 arbitrary units, corresponding to situations ranging from no damaged comets to all comets extremely damaged:

$$DD\ (au) = (0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4)/(\Sigma n /100)$$

Where DD (au): Arbitrary unit DNA damage score, n_0-n_4: number of Class 0-4 comets, Σn: total number of scored comets. Coefficients 0-4 are weighting factors for each class of comet.

Disomy assessment of sperms of all study groups using dual-color PRINS. The sperm sample was washed twice in 1× PBS by centrifugation (550 ×g for 5 min) and fixed in fresh fixative (3:1 methanol: glacial acetic acid) at 4°C for 1 hour. The sperm suspension was then dropped onto clean microscope slides and air-dried. The slides were aged for 1 day at room temperature. Before doing the PRINS procedure, the slides were immersed in a 0.5 M NaOH solution at room temperature for 4 min, passed through an ethanol series (70%, 90% and 100%) and air-dried. The use of NaOH solution allowed the simultaneous decondensation and denaturation of sperm nuclei [13]. Rapid two-color (green and red) PRINS procedure described by Yan *et al.* [24] was used to analyze chromosomes 18, 21, X and Y in all study groups with some modifications. Three slides were prepared for each sample. One slide was used for chromosomes 18 and 21; the others were used for sex chromosomes. Also, an autosome chromosome 18 was labeled (either green or red) at the same time as control with Y and X, in order to make the distinction between diploidy and disomy. For each slide, the reaction mixture was prepared in a final volume of 50 µL containing 0.2 mM dATP, dCTP, and dGTP, 0.02 mM dTTP, 0.02 mM fluorescent dUTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% BSA, 200 pmol of oligonucleotide primer, and 2.5 U of Taq DNA polymerase (All reagents from Roche Diagnostics, USA). Primer sequences used in PRINS reactions and relevant annealing temperatures are shown in Table 2. Double PRINS reactions were performed on a thermal cycler as previously described [13]. After the PRINS reaction, the slide was examined under the epifluorescence microscope (E800, Nikon, Tokyo, Japan), preferentially using first the triple or double band-pass filter, and confirming the coloration of the fluorescent spot with single band-pass filters. Slides with more than 95% fluorescent label were considered for disomy analysis.

Statistical analysis. Results were analyzed using SPSS statistical software (version 16.0, SPSS Inc., Chicago, IL, USA). The Mann-Whitney U test was

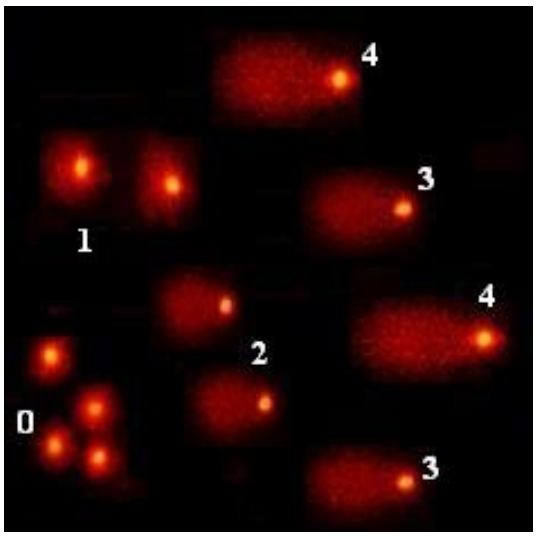

Fig. 1. Typical sperm nuclei with various degrees of DNA damage observed as comet. Score 0 denotes undamaged cell and score 4 denotes heavily damaged cell (see text for more details). All types of comets are seen in all studied groups, but the frequency of each type differed between normal and infertile groups (magnification ×400).

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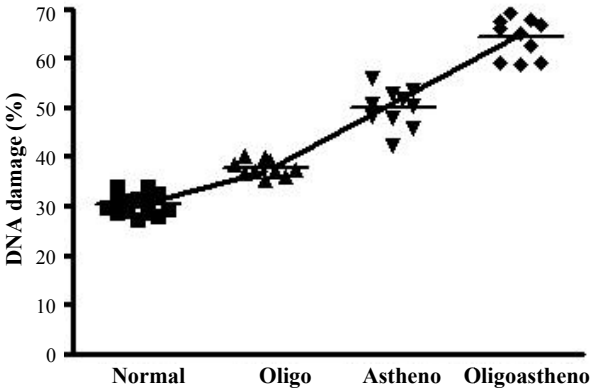

Fig. 2. Percentage of DNA damage calculated for sperm samples from normal (n = 14) and subfertile individuals (n = 10 each group). Each data–point indicates data obtained for one sample. Redrawn from Nili *et al.* [20] with permission.

used to compare differences between types of infertility. Sigma Plot 2004 for Windows (version 10.0) was used to draw figures. $P<0.05$ was considered as statistically significant.

RESULTS

DNA damages according to sperm parameters. Analysis of DNA damage was performed on sperm samples from both normal and subfertile men. Figure 2 shows the results obtained after DNA damage analysis of studied samples. As seen in normal samples, about 30 percent DNA damage was observed which increased for all samples from subfertile groups with the highest level of DNA damage (about 70%) for oligoasthenozoosperms. The data points in Figure 2 clearly indicates that the difference seen as percentage of DNA damage in different study groups was not random and was characteristic of sperms in each study group. Cluster of data points show that there was a small inter-individual difference between DNA damage calculated for each sample in the study group. There was a considerable statistical difference between DNA damage observed in normal and other study groups and also among subfertile groups ($P<0.001$).

Correlation of chromosomal disomy in sperm samples with DNA damage. For analysis of chromosomal disomy in sperm samples of normal or subfertile individuals, a minimum of 5,000 sperm nuclei per chromosome was scored. An example of PRINS labeling on sperm nuclei is provided in Figure 3 showing some types of disomy observed in samples. The data are summarized and presented in Table 3. The mean disomy rates were 0.067% for chromosome 18 and 0.231% for chromosome 21. Statistical analysis showed a major difference between the frequency of disomy for chromosomes 21 and 18. There was no statistical difference ($P>0.1$) between XX and YY disomy, but the rate of XY disomy, almost three times higher, was considerably different with either XX or YY disomy ($P<0.01$).

Although disomy rates for all studied chromosomes were higher in oligozoospermia and oligoasthenozoospermia samples compared to the normozoospermia group, statistically different ($P<0.01$), results obtained for asthenozoospermia group was very much similar to normal individuals not different ($P>0.05$).

As seen in Figure 4A, the frequency of X, Y and XY disomy in oligoasthenozoospermia group is much higher than other subfertile or normal group ($P<0.01$). The mean frequency of X, Y or XY disomy in oligozoospermia, although lower than that of oligoasthenozoospermia, is higher and considerably different compared to normal or asthenozoospermia group ($P<0.05$). It might worth to mention that the rate of diploid sperms, although so rare, was not considered for analysis. The rate of sperm DNA damage with sex chromosome disomy is very much similar to the disomy rate. As the DNA damage increased, the frequency of disomy also increased except for the asthenozoospermia group (Fig. 4A).

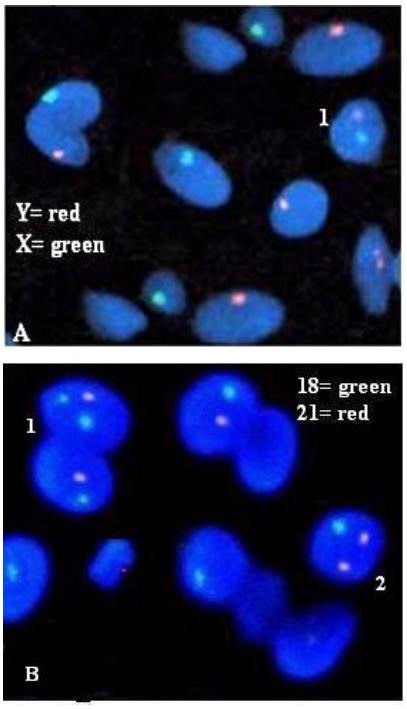

Fig. 3. Example photomicrographs of sperm nucleus showing *in situ* PRINS labeling and chromosome disomies; **A1** (XY); **B1** (18/18) and **B2** (21/21). Since the frequency of disomy was very low in either case, photomicrographs of cells showing disomy for each category were put in a single plate just to show the appearance of labels on each sperm. The appearance of labels in sperms of all studied group was similar (magnification ×1000).

Table 3. Frequency of disomy % (mean ± SD) assessed by primed *in situ* labeling (PRINS) technique in sperm samples from normal and subfertile men. Fourteen subjects in normal group and ten subjects in each subfertile group were studied for the presence of disomy. Errors are standard error of mean values.

Sperm samples	No. of subjects	1818	2121	XX	YY	XY
Normal	14	0.067 ± 0.013	0.231 ± 0.064	0.066 ± 0.015	0.059 ± 0.020	0.184 ± 0.047
Oligozoospermia	10	0.117 ± 0.039	0.331 ± 0.058	0.126 ± 0.028	0.076 ± 0.020	0.320 ± 0.050
Asthenozoospermia	10	0.068 ± 0.012	0.261 ± 0.043	0.073 ± 0.013	0.059 ± 0.019	0.204 ± 0.042
Oligoasthenozoospermia	10	0.251 ± 0.046	0.445 ± 0.087	0.152 ± 0.025	0.127 ± 0.017	0.638 ± 0.052

The disomy rate for chromosome 18 was the highest in oligoasthenozoospermia group (Table 3) and highly different compared to other two subfertile and normal groups (P<0.01). The mean rate of disomy for chromosome 18 was higher in oligozoospermia group compared to control, and asthenozoospermia group (P<0.05). Higher frequency of disomy for chromosome 21 was observed in all studied groups (Table 3). Similar to other chromosomes, the highest rate belonged to the oligoasthenozoospermia group (statistically different <0.01). Figure 4B shows the correlation of DNA damage with sperm chromosome disomy for autosome chromosomes studied. As seen, a similar trend for increasing DNA damage and sperm disomy for chromosomes 18 and 21 was observed expect for the asthenozoospermia group. Frequency of disomy in asthenozoospermia was similar to normal group, but the rate of DNA damage was higher in asthenozoospermia group (P<0.05, Fig. 4B).

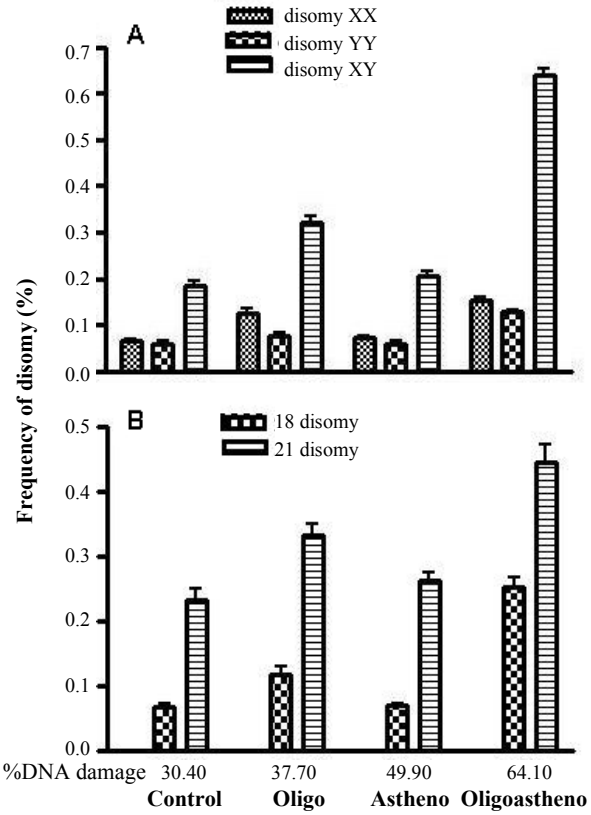

Fig. 4. Bar charts showing percentage of DNA damage versus percentage of disomy observed for chromosomes X, Y and XY **(A)** and disomy observed for chromosomes 18 and 21 **(B)**.

DISCUSSION

Valuable information on the incidence of aneuploidy in sperm from normal men has been provided over the past decade using different cytogenetic methods. Disomy levels for nearly all human chromosomes have been reported to date [25]. However, in some cases, a wide variability in the reported frequencies of disomies for the same chromosomes was noticed when data came from different research groups. These results could be explained through inter-individual differences. However, technical aspects such as differences in sperm decondensing protocols, scoring criteria, number of sperm analyzed and the characteristics of the probes used could be main factors with an influence on the results and frequencies reported [26]. Because of the high complementarities between the oligonucleotide primers and their genomic targets, PRINS appears to be a good alternative to FISH assay on human sperm when analyzing only repeat DNA sequences.

The baseline incidence of disomy for chromosomes 18 and 21 was found to be consistent with the report by Kirkpatric *et al.* [4], but differed statistically from others [27]. However, frequency of disomy of chromosomes 18 and 21 was considerably higher in sperm samples from subfertile oligozoospermia and oligoasthenozoospermia (Table 3). Disomy rate for these chromosomes in asthenozoospermia samples was fairly similar to samples retrieved from normal men, not statistically different.

Our estimate for XX and YY frequency in normal individuals (Table 3) is similar to the rates reported by Collodel *et al.* [3], but the disomy frequency of XY observed in our study was considerably different from the reports of the same authors (0.09 and 0.3).

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Kirkpatric *et al.* [4] reported a higher rate of disomy for sex chromosomes compared to our results (Table 3).

As seen in Table 3, frequency of disomy of sex chromosomes in oligoasthenozoospermia was higher than other subfertile groups especially for XY disomy which was about twice the rate of oligozoospermia and about three times more than asthenozoospermia. Observed inter-individual variation in disomy rates was not pronounced for all chromosomes. However, increased rate of sex chromosome disomy and disomy of chromosomes 18 and 21 in this group might suggest that both sexes and some autosome chromosomes may have an increased susceptibility for segregation error in spermatogenesis, or that sperm maturation is more tolerant of errors in segregation involving sex and some autosome chromosomes. This observation might be in contrast to the arguments of Kirkpatric *et al.* [4] who suggested that sex chromosomes could be more tolerant in segregation errors than autosomes.

An increased rate of sperm disomy and diploidy has been reported for patients with poor semen quality [28]. The influences of both severe oligozoospermia and teratozoospermia on sperm aneuploidy are generally accepted [29]; whether sperm aneuploidy might also occur in cases of isolated asthenozoospermia is still being debated [30]. Our observation for disomy of chromosomes 18 and 21 as well as sex chromosomes is somehow different from the results reported previously [3].

Numerical chromosomal aberrations in sperm are due to non-disjunctional events during meiosis with unknown causes. However, there are reports that indicate sperm DNA damage might be an indicator of aneuploidy [31]. DNA damage in normal and subfertile patients and also a high degree of DNA damage in oligoasthenozoospermia patients (Fig. 2) may be due to various processes including incomplete protamination [21], incomplete removal of apoptotic cells, incomplete repair during meiosis, increased aging and oxidation of spermatozoa during passage and storage in the male tract and ROS. Although various mechanisms of DNA damage in male germ cells and spermatozoa are proposed [32], ROS have received special attention due to their important role in both the physiology and pathology of human reproduction [33].

DNA damage detected by comet assay in male germ line clearly measure both single and double strand DNA breaks [34]. DNA damage especially double strand breaks (DSB) in dividing cells in spermatogenesis cycle, if induced in a single chromosome may convert to chromatid breaks. Signaling of a single DSB triggers the cell to make genomic rearrangement at the cross over point of a looped chromatin domain, possibly a transcription factory. Rearrangements may also occur between sister chromatids leading to an inversion adjacent to the break sites [35]. Completion of this rearrangement may lead to a transmissible inversion. It is also possible more than one chromosome suffer from DSB which may lead to a translocations that affect chromosome pairing and meiotic segregation. As shown in Figure 4 higher rate of DNA damages is correlated to higher frequency of chromosomal disomy for both autosome and sex chromosomes except for asthenozoospermia samples. The plausible explanation for this difference might reside in the event that spermatozoa in asthenozoospermic men suffer DNA damage after completion of the process of chromosomal segregation.

Spermatozoa are particularly susceptible to ROS-induced damage due to presence of large quantities of polyunsaturated fatty acids and low concentrations of scavenging enzymes in their plasma membranes [33]. Therefore, most of DNA damage leading to chromosomal aneuploidy in oligozoospermia and oligoasthenozoospermia patients might have been induced prior to chromosomal segregation. This statement might be supported by the experimental evidence shown by Mozdarani and his colleagues [36, 37] who studied the frequency of aneuploidy and micronuclei in preimplantation embryos produced by male mice irradiated at various stages of spermatogenesis. These authors have shown that radiation induced DNA damage in more sensitive cells in spermatogenesis i.e., spermatogonia and spermatocytes led to more chromosomal aneuploidy [36] or micronuclei [37] in preimplantation embryos generated by sperms produced by these cells. These observations might imply the involvement of DNA damage in chromosomal segregation during meiosis leading to aneuploid sperms. In a recent report, Perrin *et al.* [38] have also shown that the DNA fragmentation rate depends on the presence of a chromosomal abnormality in spermatozoa.

In conclusion, the results of the present study suggest that DNA damage might contribute to chromosomal aneuploidy in spermatozoa in normal and subfertile individuals and also an indicator of aneuploidy. The molecular cytogenetic investigation of human sperm could be efficiently used to investigate some epidemiological aspects of meiotic non-disjunction in male gametes.

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